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Venezuelan equine encephalitis virus entry mechanism requires late endosome formation and resists cell membrane cholesterol depletion

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Abstract

Virus envelope proteins determine receptor utilization and host range. The choice of receptor not only permits specific targeting of cells that express it, but also directs the virus into specific endosomal trafficking pathways. Disrupting trafficking can result in loss of virus infectivity due to redirection of virions to non-productive pathways. Identification of the pathway or pathways used by a virus is, thus, important in understanding virus pathogenesis mechanisms and for developing new treatment strategies. Most of our understanding of alphavirus entry has focused on the Old World alphaviruses, such as Sindbis and Semliki Forest virus. In comparison, very little is known about the entry route taken by more pathogenic New World alphaviruses. Here, we use a novel contents mixing assay to identify the cellular requirements for entry of a New World alphavirus, Venezuelan equine encephalitis virus (VEEV). Expression of dominant negative forms of key endosomal trafficking genes shows that VEEV must access clathrin-dependent endocytic vesicles for membrane fusion to occur. Unexpectedly, the exit point is different from Old World alphaviruses that leave from early endosomes. Instead, VEEV also requires functional late endosomes. Furthermore, unlike the Old World viruses, VEEV entry is insensitive to cholesterol sequestration from cell membranes and may reflect a need to access an endocytic compartment that lacks cholesterol. This indicates fundamental differences in the entry route taken by VEEV compared to Old World alphaviruses.

Keywords: Venezuelan equine encephalitis virus; Virus entry; Membrane fusion; Endocytosis; Late endosome; Cholesterol; Alphavirus

Introduction

VEEV is endemic to regions of South America, but is normally maintained in a silent enzootic cycle between *Culex* mosquitoes and rodents. However, sporadic outbreaks involving hundreds of thousands of people have been reported since 1938. These result from transmission of the virus to equines (horses, mules and donkeys) where it becomes efficiently amplified. Human infections via *Aedes* mosquitoes then follow (Weaver et al., 1996). Fatality in equines ranges from 38 to 80% and represents a heavy burden on the agricultural industry. Most humans succumb to a debilitating, acute febrile illness with rapid onset, lasting up to 2 weeks, and results in severe leucopenia, increasing susceptibility to secondary infection. Children and pregnant women are at greater risk, with 1 in 25 children developing neurological sequelae, that sometimes lasts for life. In 1971, VEEV crossed over the Mexican border into Texas, resulting in the deaths of 200,000 equines in Mexico and Texas before the disease was controlled by massive veterinary vaccination. With both mosquito vectors resident in North America, increased insecticide resistance in mosquitoes (Pietrantonio et al., 2000) and increases in population density (Schmidt, 2000), similar outbreaks are inevitable. Presently, there is no FDA-certified vaccine for VEEV, and the attenuated military vaccine has poor efficacy (Russell, 1999). Drugs such as Ribavirin, which are useful against other RNA viruses, are ineffective against VEEV (Canonico et al., 1984). Our knowledge of the mechanisms of pathogenesis for VEEV is extremely limited, and a more detailed understanding of the host–virus interaction will be required for the development of new therapeutics and diagnostic tools.

VEEV is a member of the family Togaviridae, genus alphavirus. It has a similar structure and genomic organization to those of other alphaviruses, including Sindbis (SINV), Ross River, Eastern Equine Encephalitis and Semliki Forest virus

Abbreviations: VEEV, Venezuelan equine encephalitis virus; MLV, murine leukemia virus; env, envelope protein.

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(SFV). Virus particles are 60–70 nm in diameter, consisting of an 11.5-kb RNA genome encapsulated in an icosahedral capsid and coated by a lipid membrane derived from the cell at the time of budding. Embedded in this membrane and non-covalently associated with the underlying capsid are the envelope proteins (envs) E2 and E1 (Paredes et al., 2001). These structural proteins are made as a single polyprotein together with capsid which cleaves itself from the envs soon after synthesis. The envs are cleaved apart by furin and from their signal peptides (E3 and 6K) by host signal peptidases (Strauss and Strauss, 1994).

The envs define the majority of cell tropism for enveloped viruses by determining receptor specificity and, thereby, the entry route into the cell. For the pH-dependent alphaviruses, trafficking to endosomes is also controlled by the envs, where a necessary drop in endosomal pH induces conformational changes in the envelope protein that promote fusion of the virus and cell membranes. By expressing dominant negative genes that inhibit endosome formation, Influenza A was found to require late endosomes for infection of cells, whereas vesicular stomatitis and SFV (an Old World alphavirus) required early endosomes that are less acidic than late endosomes (Sieczkarski and Whittaker, 2003). The virus thus regulates its endocytic release by setting the pH-threshold at which the env undergoes its conformational change. Mutations in envs that alter this threshold severely abrogate virus infectivity (Gething et al., 1986), indicating that the release point is crucial in establishing infection.

The Old World alphaviruses, such as SFV and SINV, dominate our knowledge of the alphavirus infection cycle, whereas little is known about the highly virulent New World alphaviruses such as VEEV, especially pertaining to entry. Since many examples exist of closely related viruses adopting distinct entry pathways, for example picornaviruses enter cells by clathrin-mediated endocytosis or caveolae and lipid rafts (Pietiainen et al., 2004), it is important to determine if similar trends exist for the alphaviruses. Indeed, it has been suggested that residue differences between the envs of VEEV and SFV may define dependence on cholesterol for membrane fusion in model membranes and insect cells depleted of cholesterol (Lu et al., 1999). However, cholesterol dependence has never been tested in mammalian cells. Here, we demonstrate the usefulness of a novel contents mixing assay originally described for murine leukemia viruses (Kolokoltsov and Davey, 2004) to study the entry of VEEV-env pseudotyped viruses in real time and at low MOI. Using dominant negative mutants and drug inhibitors of the endocytic trafficking pathway, we directly show that VEEV enters cells from the late endosomes and entry resists cholesterol depletion differing from SFV which exits from early endosomes and is more dependent on free membrane cholesterol. This indicates an entry mechanism that is distinct from the Old World alphaviruses.

Results

Application of a novel contents mixing assay to alphaviruses

Cellular events that lead to enveloped virus entry are poorly understood, principally due to a lack of quantitative and sensitive assay systems that can measure entry in real time. For alphaviruses, work has focused on the Old World alphaviruses, SFV and SINV as each grows to high titer and is non-pathogenic to humans. For both, infection of mammalian cells can be triggered by a drop in pH. This has been shown using lipid mixing assays, which measure the dispersion of fluorescently labeled probes from virus membranes into cell or artificial target membranes, upon fusion (Smit et al., 1999; White and Helenius, 1980). However, these assays require large amounts of purified virus making them difficult to perform with highly virulent viruses, such as VEEV. Previously, we reported a new entry assay for eco-MLV (Kolokoltsov and Davey, 2004) that allowed accurate measurement of virus entry. Here, we demonstrate that this assay can be adapted to alphaviruses and used to study VEEV entry into mammalian cells.

The basis of the entry assay is the encapsulation of luciferase enzyme directly into the virus particle. Only when virus–cell membrane fusion takes place can the enzyme access its substrates and generate a signal. Recently, we reported the production and characterization of high-titer, VEEV env-pseudotyped MLV (Kolokoltsov et al., 2005), and demonstrated that these particles enter cells in a VEEV env-dependent manner. Epitopes normally presented on wild-type envs were also detected on particles and neutralized these particles identically to wild-type virus. These pseudotypes are generally accepted to adopt the entry mechanism and pathway of the env donor but importantly do not replicate and provide the opportunity to study the entry of dangerous viruses in low level containment (Sharkey et al., 2001; Simmons et al., 2003).

We adapted the VEEV envs to accept the luciferase enzyme as for the eco-MLV env-based system. For this, we modified the E1 protein of VEEV at the c-terminus to make a fusion with luciferase (Fig. 1A). An extended linker peptide using 3-copies

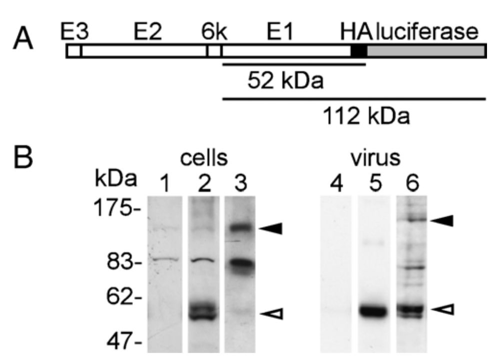

Fig. 1. Construct design and env-luc expression. (A) Recombinant env gene was made in the mammalian expression vector pCDNA3 (Invitrogen). The single ORF encoded the env of VEEV from E3 through E1 as previously reported (Kolokoltsov et al., 2005), except the termination codon was removed and fused to a gene encoding three copies of the HA epitope (HA) which, in turn, was connected to the firefly luciferase gene. (B) Expression of the recombinant VEEV env gene was examined when making MLV particles by a Western blot of lysates of transfected cells (left) and pelleted virus (right). Cells were transfected to make MLV particles (as described in Materials and methods) pseudotyped with env from eco-MLV (lanes 1 and 4), VEEV env with HA-tag (lanes 2 and 5) or the VEEV env-luc construct (lanes 3 and 6). Open and solid arrowheads indicate predicted size of VEEV HA-tagged E1 protein and E1-luc proteins, respectively.

of an HA epitope peptide (Davey et al., 1997) was required to produce virus-associated luciferase (Fig. 2). Furthermore, the HA-tag also provided a simple way to detect the E1 protein by Western blot (Fig. 1B, open arrowhead). The E1-luciferase fusion protein was evident as an approximately 100-kDa band on Western blots of cells (lane 3) and virus particles (lane 6, solid arrowhead).

Virus particles were then characterized on continuous sucrose gradients. Filtered culture supernatants were applied to gradients, and fractions were collected. Virus titer, luciferase activity after lysis and E1 reactivity by Western blot analysis were measured. For each measurement, activity peaked in fraction 4, corresponding to approximately 40% sucrose (Fig. 2). In our previous work, we used the ratio of luciferase signal from lysed versus unlysed particles to gauge signal to noise. From previous work, a ratio of >5 indicated that particles were intact and would give a good measure of entry after membrane fusion. Our VEEV construct gave a ratio of >10. Together with the sucrose gradient data, this finding strongly indicated that the VEEV-pseudotyped particles were intact, infectious and likely to function well in an entry assay.

The particles were then tested in an entry assay. A major advantage using pseudotyped virus is that the entry mechanism of each virus can be evaluated and directly compared to other particles where only the origin of the env has been changed. Here, we used eco-MLV and VSV env particles as controls. VSV enters cells through clathrin-mediated endocytosis while eco-MLV is thought to enter at the cell surface. Cells were incubated with virus-containing luciferase for times up to 2 h at an MOI of 0.5. VEEV-env-luc, eco-MLV env-luc and VSV-G + eco-MLV env-luc-coated particles were used. For each, a signal was generated that increased over time (not shown). To obtain an accurate determination of entry kinetics, we performed a rapid-binding entry experiment. Virus was incubated for 1 min with cells, after which unbound particles were washed free by two rapid washes in DMEM. Cells and bound virus were then incubated at 37 °C for up to 1 h, samples were collected and luciferase activity measured. Eco-MLV env-

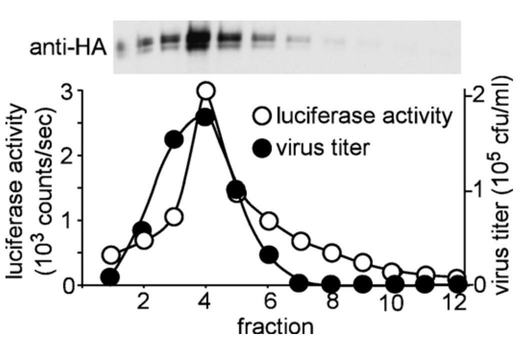

Fig. 2. Analysis of luciferase-containing virus particles. A continuous sucrose gradient (0–60% w/v) was used to separate particles and fractions were taken. Fractions were tested for the E1-HA-luciferase fusion protein by Western blot and probing with an anti-HA monoclonal antibody (12CA5, Roche) at 1:2000 dilution. The secondary antibody was anti-mouse IgG-horseradish peroxidase conjugate at 1:2000. Fractions were also assayed for virus titer using a β-galactosidase reporter assay, and luciferase activity was measured after lysis of particles in 1% Triton X-100.

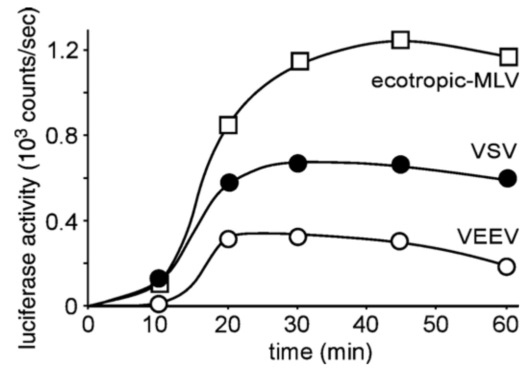

Fig. 3. Virus entry kinetics. Viruses containing luciferase were concentrated and applied to cells at an MOI of 10 for 1 min at 37 °C. Excess virus was then washed free of cells by two washes and pelleting cells with remaining bound virus (<5% of innoculum) for 1 min at 400 × g. The cells were then resuspended in DMEM and incubated at 37 °C. At times indicated, samples of 10^5 cells were taken and luciferase activity was measured (as described in Materials and methods). The entry of MLV particles pseudotyped with envs of VEEV, eco-MLV and VSV + eco-MLV was analyzed.

coated particles were assayed on 293 cells expressing a recombinant form of the eco-MLV receptor. Others were assayed on 293 cells. Since we are measuring entry kinetics in real time, we have found that small changes in incubation conditions can greatly affect the measurements. In our previous work, we reported that eco-MLV entered with a half-time of 40 min (Kolokoltsov and Davey, 2004). We have since observed that eco-MLV is very temperature sensitive and use of an air incubator gave poor heat transfer to the cells. Using a waterbath gave a more accurate half-time of entry of 20 min for eco-MLV. The entry of VSV-G- and VEEV env-bearing particles was more rapid, with half-times of 15 and 10 min, respectively (Fig. 3) and not as temperature-dependent (data not shown). These kinetics are consistent with a rapid entry pathway for VEEV, possibly that of clathrin-mediated endocytosis.

To further test the specificity of the entry signal, virus was preincubated with VEEV-reactive antibodies before application to cells. The signal from the VEEV particles was reduced by >80%, whereas VSV particles were unaffected (Fig. 4, upper). This indicated that the particles entered cells by a VEEV env-mediated mechanism.

Old World alphaviruses enter cells through a pH-dependent mechanism. To determine if VEEV entry uses this mechanism, cells were treated with inhibitors of endocytic acidification and then luciferase entry assays were performed. Eco-MLV (a pH-independent virus) env-bearing particles served as a control for cell viability (Kolokoltsov and Davey, 2004). The inhibitors bafilomycin, ammonium chloride, chloroquine and monensin were used. Each effectively blocked the entry signal for VSV (a pH-dependent virus) env-bearing particles and decreased the VEEV entry signal similarly. In contrast, the eco-MLV was unaffected indicating that cells were competent for mediating virus entry and that the kinetics of the luciferase-based measurement had not been altered (Fig. 4, lower). Together, these data indicated that VEEV particles enter cells by a pH-dependent mechanism, and that the luciferase-entry-assay reflected the entry pathway used by infectious virus validating

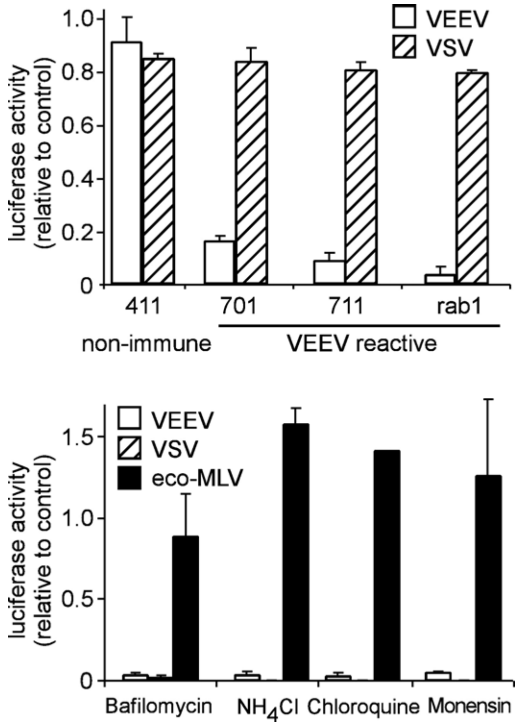

Fig. 4. Neutralization of VEEV particles by virus-specific antibodies and effects of inhibitors of endosomal acidification. For antibody neutralization (upper panel), virus was incubated with the indicated antibody for 1 h at a dilution of 1:200. Cells were then added and incubated for 1 h at 37 °C and then pelleted by centrifugation at 400 × g, and luciferase activity was measured (as described in Materials and methods). Antibody 411 is preimmune serum from the rabbit used to make VEEV-specific antisera #711. 701 is a second immune rabbit serum from ATCC. Rab1 was a rabbit hyperimmune antisera against VEEV provided by Dr. Weaver, UTMB. A VSV-G pseudotyped MLV was used to detect non-specific neutralization of virus. The effect of inhibitors of endosomal acidification was also tested (lower panel). Each inhibitor (indicated) was incubated with cells for 1 h prior to addition of virus (MOI of 0.1) and throughout the 30 min incubation with virus. Results are the average of 3 independent experiments, and data were normalized to signals obtained from untreated or solvent controls. Inhibitors used were Bafilomycin A1 (50 nM, final concentrations given), Ammonium chloride (NH_4Cl, 20 mM), chloroquine (100 μM) and Monensin (1 μM).

the assay as a useful tool in further analyzing the entry mechanism of VEEV.

Little is known about the entry route of New World viruses, such as VEEV. As shown above, an endocytic pH-drop is an important step in VEEV entry. However, a number of endocytic pathways have been identified that become acidified, and many inhibitors do not discriminate between these (Johannes and Lamaze, 2002). In addition, drugs such as Bafilomycin can indirectly affect the trafficking of endocytic vesicles (van Weert et al., 1995). Ectopic expression of dominant negative (DN) mutant forms of genes important in endocytic trafficking provides an alternative means to block key endocytic steps. We chose DN forms of Eps15, Rab5 and Rab7 as each blocks a specific step in endocytic trafficking and tested each in the entry assay for its impact on VEEV entry. The mutant form of Eps15, EΔ95/295, has a 200-amino acid deletion that prevents association with AP2, a key mediator of clathrin-mediated endocytosis and inhibits this pathway. Rab5 S34N and Rab7 T22N block endocytic vesicle trafficking to the early and late endosomes, respectively (Feng et al., 2001). Rab5 Q79L is a constitutively active form of Rab5 and causes accumulation of early endosomes (Roberts et al., 1999). A major drawback in using these genes is they need to be introduced into cells by transfection using expression constructs. We found that this resulted in highly variable expression, complicating any quantitative measurement (Fig. 5A, right). To overcome this obstacle, each gene was subcloned into a lentiviral packaging plasmid and used to produce lentiviruses. The use of lentivirus vectors is advantageous, as the transgene is permanently integrated into the cell chromosome, and gene transcription is driven by normal cellular transcription machinery with no viral genes present. Additionally, the expression levels can be controlled by the MOI of lentivirus infection. Each DN gene, tagged with GFP, was introduced into 293 cells using lentivirus vectors and expression profiles analyzed by FACS (Fig. 5B). At an MOI of 5, >95% of the cells expressed the transgene within 5-fold of the mean, which was 100-fold above background values defined by vector without insert. The exception was EΔ95/295 that gave consistently low level expression. However, as noted below, the gene product functioned well, indicating that the low fluorescence signal may be due to high protein turnover. Given the generally high efficiency of transgene expression, the entry assay measurements should

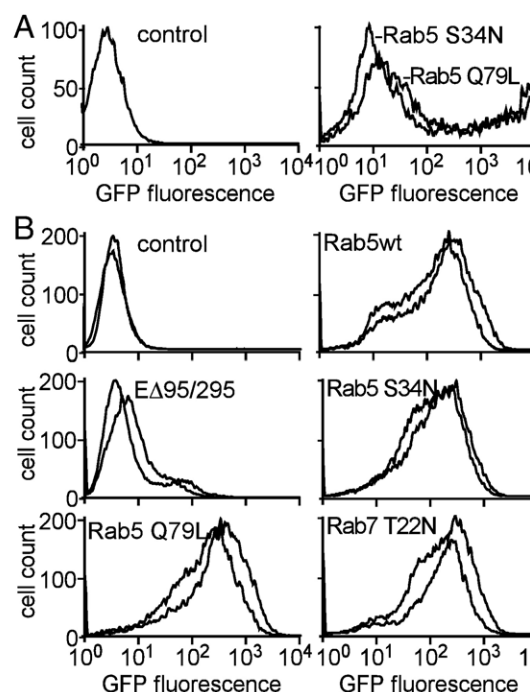

Fig. 5. FACS analysis of expression of DN genes following transfection or delivery by lentiviral vectors. (A) Cells were transfected using calcium phosphate-mediated transfection with plasmids encoding the indicated GFP-tagged constructs. Expression levels were then monitored by FACS analysis. 20,000 events were counted for each sample. Expression levels were seen at a low (majority) and high level. (B) Cells were transduced with lentivirus expression vectors encoding the indicated GFP-tagged constructs at an MOI of 5. Expression profiles were then determined by FACS analysis. As compared to the calcium-mediated transfection, a more uniform profile of expression was seen for each. Two independent experiments are shown for each.

reflect the overall effect gene expression on the entire cell population.

The effect of the clathrin-pathway blocking DN mutant of Eps15, EΔ95/295, was examined first. As a control, the impact of each trafficking gene mutant was monitored by observing transferrin uptake, which occurs strictly through the clathrin-mediated endocytosis. Cells were incubated with Alexafluor$_{594}$-transferrin and, after fixing, the distribution of the gene product and the labeled transferrin was assessed by confocal microscopy (Fig. 6A). In untreated cells, label was rapidly taken up into the cytoplasm with punctuate staining (Fig. 6, control). However, in cells infected with the EΔ95/295 encoding lentivirus, the transgene product was weakly but evenly distributed throughout the cytoplasm, while the labeled transferrin was found only on the surface and within a small region near the nucleus. This indicated that clathrin-mediated endocytosis had been effectively inhibited. Entry was then measured for VEEV, VSV and eco-MLV env-pseudotyped virus. VEEV and VSV behaved similarly with >70% block in entry observed (Fig. 6B, open and solid bars), while eco-MLV was not affected (Fig. 6B, striped

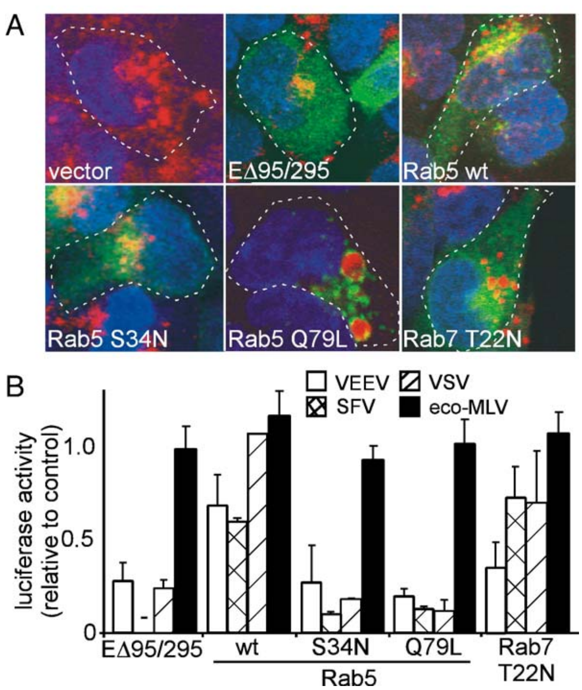

Fig. 6. The effect of expressing mutant forms of proteins important in endocytosis on virus entry. Mutant endocytic trafficking genes (GFP-tagged) were delivered in lentivirus expression constructs, and after 36 h, the effect of each on transferrin uptake (A) or virus entry (B) was determined. The effect of ectopic expression of each gene on clathrin-mediated endocytosis was measured by observing Alexafluor$_{594}$-labeled transferrin (red) uptake by confocal microscopy. Transgene products were localized by GFP fluorescence (green). Vector alone (carries blasticidin resistance marker) was used as a control (titered by drug selection). Outlines of representative cells expressing the transgene are drawn in with dashed lines. Nuclei were stained with DAPI (blue). For virus entry assays, MLV pseudotyped with envs from VEEV, SFV, VSV or eco-MLV and containing luciferase enzyme was made (as described in Materials and methods). These viruses were incubated with cells for 1 h at 37 °C, and then entry signals were determined by measuring luciferase activity. Data are an average from 3 independent experiments and signals were normalized to that from cells infected with the vector alone.

bars). This confirmed that, like VSV, VEEV required clathrin-mediated endocytosis for triggering of entry.

Clathrin-dependent vesicles are trafficked and fuse to early and late endosomes, becoming progressively more acidic. We next examined the role of early and late endosomes in triggering VEEV entry. DN mutant forms of Rab5 (S34N) and Rab7 (T22N) were used. As for EΔ95/295, each was made as a fusion protein with GFP to track expression (Fig. 6, upper). Transferrin uptake was used to confirm the effectiveness of expression of each mutant on clathrin-mediated endosomal trafficking. Expression of wild-type Rab5 did not appear to inhibit uptake of transferrin, but distinct overlapping of transferrin and punctate Rab5-GFP signal was observed, as expected. Expression of the DN Rab5 and Rab7 genes resulted in disruption of this pattern. Rab5 S34N resulted in weak transferrin staining similar to that observed for EΔ95/295. Expression of Rab7 T22N resulted in transferrin collecting in vesicles, but each was larger and less numerous than those seen with wild-type Rab5. A similar effect was described when this mutant was overexpressed in HeLa cells (Feng et al., 2001). The constitutively active mutant, Rab5 Q79L, was the most profound, giving rise to large transferrin-containing vesicles bounded by the GFP fusion protein. These likely represent accumulation of endocytic vesicles at the early-endosome stage of maturation that remain coupled to the mutant Rab5 (Roberts et al., 1999). In each case, these phenotypes indicated that the expression vectors were driving adequate expression levels of each DN gene to obtain the desired phenotype.

VEEV entry was then measured, with VSV and eco-MLV env pseudotypes used as controls. A pseudotype of SFV was included to permit direct comparison of VEEV entry to entry of Old World viruses. The SFV construct was modified for use with the luciferase entry assay (Saeed et al., submitted for publication). VSV and SFV have been reported to exit the clathrin-mediated endocytic pathway at the early endosome, while eco-MLV is thought to enter cells at the plasma membrane. Since the only difference between each of the pseudotyped viruses is the env presented on their surfaces, differences in the entry kinetics are solely a function of the envs. For each of the genes expressed, MLV entry remained unaffected (Fig. 6, lower), confirming that the cells remained susceptible to virus entry and that ectopic expression of the mutant genes did not adversely affect the luciferase assay measurements. In contrast, expression of EΔ95/295, Rab5 S34N and Rab5 Q79L reduced the entry of VEEV, SFV and VSV to a similar extent, i.e., by 80–90%. The ability of Rab5 Q79L to inhibit virus entry to a similar extent likely reflects virus being held in the accumulated early endosomes at a step prior to reaching the required acidification for triggering membrane fusion. Overexpression of wild-type Rab5 reduced VEEV and SFV entry similarly, by 30%, but did not affect VSV entry kinetics. The effect of ectopic expression of wild-type Rab5 on VEEV and SFV may reflect increased trafficking of virus into a non-productive Rab5-mediated pathway that is still productive for VSV. VEEV and SFV behaved differently when Rab7 T22N was expressed in cells. VEEV entry was reduced by Rab7 T22N to a similar extent seen with DN Rab5 and EΔ95/

295. In contrast, SFV and VSV entry was reduced by only 20%. Together, these data are consistent with an entry route by which VEEV is endocytosed through clathrin-mediated endocytosis but entry is triggered in the late endosome, being affected by blocks that lead up to and including this step.

The finding that the VEEV exits from late endosomes indicates that the entry mechanism of this virus may be different from the Old World alphaviruses. Previous work has demonstrated that SFV and SINV require cholesterol for efficient membrane fusion in model membranes lacking cholesterol and insect cells depleted of cholesterol. The residues responsible for cholesterol dependence were identified by isolating mutants that were less dependent on the presence of cholesterol (Lu et al., 1999; Vashishtha et al., 1998). Interestingly, some of the residue changes identified are already present in the E1 env of VEEV. The prediction that VEEV may enter by a cholesterol-independent mechanism may indicate a further fundamental difference between the Old and New World alphavirus entry pathways and indicate a need to access an endocytic compartment where cholesterol is lacking. To test this, we used nystatin to sequester cellular cholesterol. Similar treatments of cells have been previously shown to block the cholesterol-dependent entry of Ebola (Empig and Goldsmith, 2002). A modification of the entry assay permitted use of Ebola env particles as a control for the entry assay (Saeed et al., submitted for publication). Cholesterol-dependent uptake of labeled cholera toxin B-subunit was also used to control for effective cholesterol depletion by disruption of its cholesterol-dependent uptake into cells (Orlandi and Fishman, 1998). The minimum dosage of nystatin that disrupted cholera-toxin subunit uptake without affecting long-term cell viability was 60 μg/ml. At this dosage, Ebola entry was blocked by >90%. Entry of VSV and SFV was also affected significantly, being inhibited by 55 and 65%, respectively (Fig. 7). This contrasted to VEEV that showed marked resistance to nystatin treatment and entry being impaired by only 20%. This indicates that VEEV enters by a mechanism that is independent of free-cholesterol, making it distinct from the Old World alphaviruses.

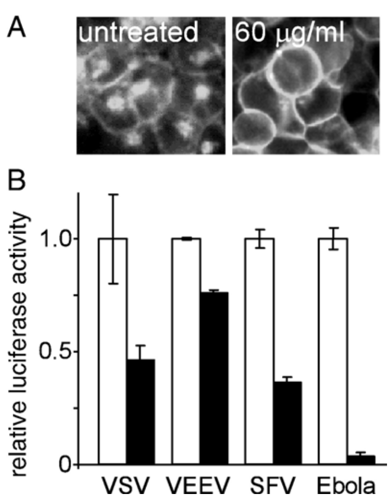

Fig. 7. VEEV entry is insensitive to cell membrane cholesterol sequestration. (A) Human fibroblasts (HEK 293 cells) were treated with 60 μg/ml nystatin for 1 h and then were stained with $Alexa_{568}$-labeled cholera toxin subunit B for 30 min. The uptake of cholera toxin is dependent on free membrane cholesterol (Orlandi and Fishman, 1998) and the minimal dosage required to inhibit uptake (staining present on the cell surface but little internal staining) was used for virus entry assays. (B) For entry assays, pseudotyped particles with envs of VEEV, SFV, VSV or Ebola were used. Virus entry was measured in untreated (open bars) or cells treated with 60 μg/ml nystatin (solid bars) by measuring luciferase activity after 1 h. Values were normalized to the vehicle (DMSO) treated control. Averages of two independent experiments are given.

Discussion

Luciferase-based contents mixing assay for VEEV

In our previous work, we reported the development of a novel contents mixing assay for measuring the fusion of virus and cell membranes (Kolokoltsov and Davey, 2004). This was based on the concept that luciferase encapsulated within the viral envelope remains inactive until it is exposed by a virus–cell membrane fusion event, and the luciferase substrates (luciferin, ATP and oxygen) can access the enzyme. The assay provides advantages over previous methods that require fluorescent dye addition to viral and/or cell membranes, detection of viral genetic material release and direct microscopic detection of fusion events. The assay sensitively and quantitatively measures events very early in the infection process and provides real-time kinetic measurements of the entry process. The VSV and VEEV entry kinetics that we have obtained compare favorably with those obtained using conventional fluorophore dequenching assays, with half times of 15 min for each used for VSV, SFV and SINV (Blumenthal et al., 1987). Since an MOI of <1 was used in our experiments, as compared to 10–100 for dequenching assays, this finding indicates little virus cooperativity in alphavirus entry.

Our findings show that the assay can be used to follow the entry of class II enveloped viruses. These viruses, which include flaviviruses and alphaviruses, have envs with distinctly different structures to the envs of class I viruses such as MLV for which the assay was originally developed. The luciferase gene was fused directly to the 3′ end of the VEEV E1 gene, likely placing the enzyme into the lumen of the virus particle. For this, a 30-residue HA epitope peptide was required to obtain particles that were initially impermeable to the luciferase substrates, as indicated by the >10-fold increase in luciferase activity, upon particle lysis in detergent. The added advantage of using the HA epitope linker peptide was that we were able to detect E1 expression on Western blots by a commercially available anti-HA monoclonal antibody. From this work, it seems that three important factors contribute to successful adaptation of the assay to new viruses. Firstly, the peptide tethering the luciferase to the env may require customization. Secondly, constructs that worked for both MLV (Kolokoltsov and Davey, 2004) and VEEV showed the luciferase being partly cleaved off the env by an unidentified protease(s). This, in turn, achieves two outcomes: very little luciferase is actually taken into particles, and the luciferase is free to separate from the viral membrane after a fusion event. Thirdly, when making the viruses, it is important to add the recombinant env-luc construct together with an excess of the wild-type env construct, in a ratio of about 1:5. Env-luc by itself results in defective particle formation, as particles become permeable to luciferin. Taking these factors into consideration, it should be possible to use this system to measure entry for many other enveloped virus types. The assay should readily be adapted to other established alphavirus pseudotypes such as Ross River virus.

Mapping the entry pathway of VEEV

To date, many virus pseudotypes have been produced and in each case these recapitulate the tropism of the envelope donor virus. The VEEV pseudotypes used in this study were thoroughly characterized in previous work by neutralizing antibodies and entry inhibitors and shown to infect cells identically to the parent virus (Kolokoltsov et al., 2005). The pseudotypes provide an important advantage over wild-type VEEV as they do not replicate and can be used safely in low level containment. Here, we determined the entry kinetics of the VEEV pseudotype and found that it closely reflected that of the wild-type virus. Again, this supports the conclusion that the pseudotype enters cells by a mechanism identical to that of the parent virus.

Our measurements indicate that VEEV entry is very rapid, reaching a plateau within 20 min after contact with cells. For all of the viruses studied in our work, we have noticed that a lag of approximately 10 min precedes the detection of an entry signal. Since all of the kinetic analyses in this report were performed on prewarmed cells and virus is prebound to cells, the lag must reflect important postbinding steps necessary for entry. This may represent the time required for trafficking into cellular compartments where entry is triggered. Interestingly, eco-MLV shares this sigmoidal entry kinetic. Given that MLV are generally thought to penetrate at the cell surface and, therefore not requiring endosomal trafficking (see below), the lag for MLV suggests a need to either recruit multiple receptors or enter a preendosomal membrane microdomain. The observation that eco-MLV was unaffected by DN Rab5 and Eps15 mutant expression is consistent with this conclusion.

We showed that VEEV enters cells by a pH-dependent mechanism by treating cells with inhibitors of endosomal acidification. Ammonium chloride and chloroquine function as weak bases to buffer changes in endosomal pH (de Duve et al., 1974). Monensin is an ionophore that decouples the proton gradient in intracellular compartments (Mollenhauer et al., 1990), while Bafilomycin A1 directly blocks the endosomal ATPase-dependent proton-pump (van Weert et al., 1995). Each was a potent inhibitor of entry for the VEEV and VSV env pseudotypes. Both VSV and alphaviruses have been shown to have a strong pH-dependence, which indicated that the assay was capable of measuring the entry pathway of these viruses, which likely proceeded through an endosomal pathway. In contrast, eco-MLV entry was not inhibited. The MLV served as an important control in this case, showing that the cells were competent for virus entry and that the entry assay was not adversely affected by any of the treatments.

To more precisely determine the endosomal pathway needed to trigger the VEEV entry mechanism and the point at which entry was triggered, we used expression of dominant negative forms of genes responsible for endocytic trafficking. Since the assay measures virus entry for a population of cells, it was important to obtain even and robust expression in most cells. We used lentivirus expression vectors, as they stably integrate the transgene into cell chromosomes and drive high expression levels by strong *cis*-acting promoters. By varying the MOI, we could carefully control the level of transgene expression and obtain consistent results.

Dominant negative gene expression indicated initially that VEEV entry, like VSV, requires functional clathrin-mediated endocytosis and early endosome formation. However, VEEV differed from VSV and from its relative, SFV, as it also required functional late endosomes, making VEEV more like Influenza A than SFV and VSV. Further studies will be required to determine if this difference is due to a lower pH-threshold at which membrane fusion is triggered, as attained in the late endosome or a dependence on secondary factors present in the late endosome. A second difference was seen in the cholesterol dependence of VEEV compared to SFV. Old World alphavirus membrane fusion has been shown to be heavily dependent on cholesterol and sphingolipids in the target membrane (Lu et al., 1999; Phalen and Kielian, 1991). These studies were performed using artificial membranes and insect cells depleted of cholesterol using methyl-β-cyclodextrin. Similar studies have not been performed with mammalian cells until now. Furthermore, mutant SFV have been isolated that are less cholesterol-dependent. Amino acid substitutions responsible for cholesterol insensitivity have been mapped to the E1 env protein (Vashishtha et al., 1998). VEEV E1 already has residues that were predicted to make the virus cholesterol insensitive. We used nystatin treatment to sequester cholesterol and showed that this treatment blocked entry of the cholesterol sensitive Ebola virus (Empig and Goldsmith, 2002) and cholera toxin uptake, which is also cholesterol-dependent (Orlandi and Fishman, 1998). SFV entry was also sensitive to cholesterol sequestration albeit to a lesser extent than Ebola. In contrast, we found that VEEV entry was relatively insensitive to treatment. Our findings demonstrate that the observations made in model membranes and insect cells on cholesterol dependence also apply to mammalian cells. The difference in cholesterol requirements may reflect differences in the membrane composition of vesicles from which the virus fuses and exits into the cell cytoplasm. Indeed, the late endosomal compartment has been shown to be depleted of membrane cholesterol while early endosomes and recycling vesicles are enriched in cholesterol (Kobayashi et al., 1998, 2002). The role of cholesterol in entry of SFV may then reflect a need to exit from early endosomes while VEEV has adapted to be less cholesterol-dependent due to a need to exit from cholesterol-poor late endosomes. In addition to pH, cholesterol may then provide a novel mechanism for a virus to sense what step of maturation an endosomal vesicle has reached so that it can exit at the optimal locale within the cell for virus replication.

Use of luciferase-containing particles in rapid diagnostic and drug screening assays

The development of the rapid entry assay for an alphavirus is also useful for rapid diagnostic and drug screening assays. Neutralization assays for VEEV are still used as the primary method for detecting patient antibodies, as a marker of concurrent or previous infection, and presently use infectious virus or, more recently, reporter gene-expressing replicons. Assays involving infectious virus require biocontainment level 3 facilities, and the CDC recommends that all personnel in these facilities be vaccinated with the non-commercial military vaccine TC-83. Virus and replicon assays yield results in 48–72 and 6 h, respectively. However, it should be possible to use luciferase-containing particles to yield a quantitative measure of serum neutralization in 15 min after application of virus to cells. Similarly, drug screens to identify factors inhibiting VEEV entry could be rapidly implemented without the need for infectious virus. The assay also provides information on drug action. Both applications have advantages over current methods and will help us to identify and treat disease resulting from VEEV infection.

Materials and methods

Chemicals

Bafilomycin A1, chloroquine and monensin were from Calbiochem (San Diego, CA). All other chemicals were Sigma (St. Louis, MO) Ultragrade unless stated otherwise.

Antibodies

Polyclonal sera raised against VEEV were from ATCC. These were sera 701 and 711 (ATCC #VR-1249AF and #VR-1250AF). A third serum (rab1) was from an VEEV-infected rabbit and was provided by Dr. Scott Weaver (UTMB, TX). Non-VEEV reactive antiserum #411 was from a preimmunized rabbit used to produce serum #711 (ATCC #VR-1250CAF).

Cell lines and cultivation

All media were supplemented with penicillin and streptomycin. 293 HEK cells were used to titer virus and for entry assays and grown in DMEM supplemented with 10% heat inactivated fetal bovine serum (Gemini Bioproducts, CA). 293FT cells (Invitrogen, CA) were used to generate env pseudotyped MLV and were grown in the same medium with 0.5 mg/ml of Geneticin (Invitrogen, CA).

Expression plasmid constructs

All plasmids were prepared using Qiagen kits (Valencia, CA) or by cesium gradient centrifugation following standard methods. A pCDNA3 (Invitrogen, CA) expression plasmid containing the VEEV envelope proteins from E3 to E1 from subtype IC strain 3908 was used to make VEEV env pseudotypes (Kolokoltsov et al., 2005). Similarly, a pCDNA3 expression plasmid containing the Semliki forest virus envelope proteins was used for SFV pseudotype production and was provided by Dr. D. Sanders (Indiana University Medical School, IN) and is described elsewhere (Kahl et al., 2004). Plasmids encoding VSV-G (pVSV-G; BD Biosciences, CA) and ecotropic MLV strain Friend-57 (eco-MLV) env (pFR57) were used to make VSV and eco-MLV env pseudotypes, respectively. These plasmids were described previously (Kolokoltsov et al., 2005). An expression plasmid encoding Ebola Zaire strain GP was obtained from Dr. P. Bates (U. Penn., PA). pGAG-POL which encodes the MLV gag and polymerase was a gift of Dr. J. Cunningham (Harvard Medical School, MA). Marker gene encoding plasmids were pψ β-gal, pψ EGFP or pFB-luc (Stratagene, CA). Each encoded β-galactosidase (β-gal), enhanced green fluorescent protein (GFP) or firefly luciferase (luc), respectively, under control of the MLV LTR and virus packaging sequence.

Production of pseudotyped MLV

MLV particles bearing the env proteins of VEEV, VSV, SFV, Ebola and eco-MLV were made according to our previous work (Kolokoltsov et al., 2005). Briefly, 293FT cells (Invitrogen) were transfected using calcium phosphate (Chen and Okayama, 1987) with pGAG-POL and either pψ β-gal, pψ EGFP or pFB-luc and plasmids encoding envs. The VSV-G-encoding plasmid was used at 1 μg per transfection to limit syncytia formation in the producer cells. All others were used at 5 μg each. After overnight incubation, the medium was replaced. When virus production peaked after a total of 36 h, the supernatants were collected and filtered through a 0.45-μm cellulose acetate filter. The filtrate was used directly, or else virus was concentrated by 2 h centrifugation at 100,000 × g and the pellet used. In some experiments, virus was purified by pelleting through 20% (w/v) sucrose in 10 mM Na HEPES, pH 7.4.

Luciferase-based contents mixing assay

The basis of the rapid entry assay is to encapsulate luciferase enzyme directly into intact virus particles. Our initial work with the assay reported the construction of luciferase-containing particles for VSV-G and MLV-env pseudotypes. For both, the enzyme was taken into particles as a fusion to the c-terminus of the eco-MLV env protein. To achieve this for VEEV, the luciferase was fused to the c-terminus of the E1 envelope protein. For this, PCR-based, site-directed mutagenesis was used to replace the stop codon with an in-frame *Xho*I site. Three copies of the HA epitope tag (Davey et al., 1997) were then connected through this site, and the luciferase enzyme was then connected in-frame through a *Sac*I site at its 3′ end. The final expression construct was assembled in pCDNA3 and named pVEEV-env-luc, and the sequence of the finalized construct was confirmed.

To make viruses containing luciferase, the expression construct was transfected together with the other plasmids used to make the env pseudotyped MLV. Typically, 1 μg of pVEEV-env-luc was used per 5 μg of wild-type recombinant env-expressing vector. Viruses were treated as above for other MLV pseudotypes. Entry assays were performed on 293 cells and typically involved incubating virus-containing culture supernatants with 10^5 cells for 1 h in a 37 °C waterbath at an MOI of 1 or less. Cells were then pelleted and resuspended in luciferase assay buffer (Promega, #E2510), after which light emission was detected in a Turner biosystems model 20/20 luminometer at 10 s, 1 min and 2 min. Typically, signal rapidly increased and reached a plateau after 1 min as luciferin entered the cells. To ensure that luciferin permeated subcellular compartments, cells were frozen in a dry-ice ethanol bath and quickly thawed in a 37 °C waterbath. With this treatment, virus remained intact (impermeable to luciferase substrates) but cells became permeable to trypan blue dye.

Rapid-binding entry kinetic assay

For analysis of entry kinetics, the above method was modified to pulse cells with virus for a short time, wash off excess virus and then follow the entry of the bound virus. This was done by briefly incubating cells with virus (typically 1 min) at an MOI of 10 infectious particles per cell. During this time, <5% of the virus bound and the excess was washed off by two 1 min washes in prewarmed DMEM. The cells and remaining bound virus were incubated for times indicated, samples taken and luciferase activity measured as above.

Determination of env-pseudotyped virus titer

293 cells were infected at a confluence of 20%, and virus was titrated by serial 5-fold dilutions. Two days later, the medium was removed and infected cells were stained for β-galactosidase activity, or GFP expression was visualized by fluorescence microscopy and colonies counted.

Use of inhibitors of endosomal acidification

Ammonium chloride and chloroquine were dissolved directly in DMEM. Bafilomycin A1 was first dissolved in DMSO at 50 μM, and monensin made as a 50 mM stock in ethanol. Both were diluted in DMEM immediately before use. For the entry assay, luciferase activity was measured after 0.5 to 1 h of incubation in the presence of the drug.

Use of dominant negative (DN) forms of endocytic trafficking mediators

EΔ95/295 (provided by Dr. Benmerah at the Cochin Institute, France) is a recombinant form of the Eps15 gene and lacks the second and third N-terminal EH repeats required for interaction with AP-2 and clathrin-coated pit formation. Expression specifically inhibits clathrin-mediated endocytosis in 293 HEK and other cell types (Benmerah et al., 1999). Rab5 and Rab7 are required for early and late endosome formation, respectively. A DN recombinant form of Rab5, S34N, blocks early endosome formation. The constitutively active mutant, Q79L, of Rab5 forces accumulation of early endosomes. Both Rab5 S34N and Q79L encoding plasmids were provided by Dr. P. Stahl at Washington University Medical School. Rab7 is required for formation of late endosomes and expression of the mutant form, T22N (Dr. Wandinger-Ness, UNM), blocks this step (Feng et al., 2001). Each was tagged with GFP to follow expression by epifluorescence microscopy. For each, a non-replicating lentivirus expression vector was constructed to permit permanent, efficient and uniform delivery by adjustment of the multiplicity of infection (MOI) and overcame other transfection artifacts.

Lentivirus expression vectors

For construction of lentivirus expression vectors, genes encoding endocytic pathway factors were cloned into pLENTI6 (Invitrogen, CA) that had previously been modified to contain a GFP gene. A common strategy was used: the plasmid vector was cut with *Xho*I, and the site made blunt-ended using T4 DNA polymerase (NEB, MA) after which it was cut with *Spe*I. Each insert was cut out from parent plasmids using *Nhe*I (compatible overhang with *Spe*I) and *Hpa*I (blunt ended) and ligated into the pLENTI plasmid. VSV-G-pseudotyped lentivirus particles were made using the Virapower (Invitrogen, CA) lentivirus expression system following the manufacturer's instructions. This involved simultaneous transfection of 293FT cells with plasmids required for assembly and packaging of the GFP-DN-fusion gene in a non-replication competent retrovirus, a technique similar to that used for production of MLV (see above). Virus-containing culture supernatant was collected after 2 days (as for MLV), purified and concentrated by centrifugation through a 20% (w/v) sucrose cushion (100,000 × g) for 3 h at 18 °C, and titered on 293 cells by counting colonies of cells expressing the GFP-fusion proteins.

Analysis of ectopic gene expression

To determine expression levels and number of cells expressing each endocytic pathway gene, a portion of the cells used for the entry assays and microscopy work was analyzed by FACS using a FACSort flow cytometer equipped with CellQuest Software (Becton Dickinson, CA). Typically, 20,000 events were recorded for each sample. Confocal microscopy was performed on a Zeiss LSM 510 META in the UTMB IDOIC imaging facility using cells cultivated on Labtek 8-well glass slides (Nalgene, NY) and fixed for 20 min in a fresh solution of 4% paraformaldehyde in PBS. They were then washed with PBS and covered with mounting medium containing DAPI (Prolong gold antifade, Molecular Probes, Invitrogen, CA). Transferrin uptake was used to demonstrate the activity of the ectopically expressed mutant genes (Rabs and Eps15) and as a measure of clathrin-mediated endocytic trafficking. When performing transferrin uptake measurements, cells were stained with 50 μg/ml of $Alexafluor_{594}$-transferrin in DMEM supplemented with 1% (w/v) BSA for 1 h at 37 °C. Excess transferrin was removed with two DMEM washes, and the cells were fixed with paraformaldehyde.

Cholesterol sequestration

Nystatin was dissolved in DMSO and cells were treated with 60 μg/ml, which was the minimum dosage that disrupted cholesterol toxin uptake, indicating that cholesterol had been effectively sequestered. This was visualized by staining of cells for 1 h with $Alexa_{568}$-labeled cholera toxin B (Molecular probes, CA). Labeling was visualized by epifluorescence microscopy using a Leica DM IRB inverted microscope fitted with a Optronics Magnafire camera. Virus entry was monitored by luciferase activity as described above.

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